

# Activation of hypolipidaemic drugs to acyl-coenzyme A thioesters

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Compounds possessing the characteristics of CoA thioesters of the hypolipidaemic peroxisome proliferators clofibric acid, nafenopin and ciprofibrate were formed on incubation of the drugs with rat liver microsomal fractions, ATP and CoA. The reactivity of the drugs correlated with their pharmacological potency. It is proposed that the active species of these compounds are their acyl-CoA thioesters.

## INTRODUCTION

The hypolipidaemic drug clofibrate and its congeners, along with other hypolipidaemic drugs of diverse chemical structure, are known to cause hepatomegaly and peroxisome proliferation in experimental animals (for a review see Cayen, 1983). In addition, these drugs induce various metabolic effects in the liver, and they have been proposed as a new class of chemical carcinogens in rats and mice, on the basis of long-term studies (Reddy *et al.*, 1980). Although some of these drugs are widely used in the prevention of atherosclerosis, the mechanism of the drug-induced hypolipidaemia, peroxisomal proliferation, and other metabolic effects, is unclear. The only common chemical characteristics shared by these compounds appear to be their hydrophobicity, and the frequent presence of a carboxylic acid functional group, or a group that can be readily oxidized to carboxylic acid.

These considerations prompted us to test the possibility that hypolipidaemic drugs could be activated to acyl-CoA thioesters by acyl-CoA synthetase. Since the bulk of this enzyme activity is located in microsomal fractions (Krisans *et al.*, 1980; Bronfman *et al.*, 1984), this subcellular fraction was used as source of the enzyme.

## EXPERIMENTAL

H.p.l.c. separations were accomplished on a  $\mu$ Bondapak C18 column (30 cm  $\times$  0.4 cm; Waters Associates, Milford, MA, U.S.A.). The liquid chromatograph consisted of a model M-45 pump, a U6K syringe-loading sample-injection valve, and a model 441 fixed-wavelength spectrophotometric detector purchased from Waters Associates. The composition of the mobile-phase solvents used in the separations and other experimental conditions are given in the legends.

Microsomal fractions were prepared from the liver of male Sprague–Dawley rats (180–250 g body wt.) as described by de Duve *et al.* (1955). They contained 85–90% of the homogenate activity of the microsomal marker NADPH:cytochrome *c* reductase, 70–75% of palmitoyl-CoA synthetase, and less than 4% of the mitochondrial marker glutamate dehydrogenase. These enzymes were assayed as described or referenced in Bronfman *et al.* (1984). Drug incubations were performed in a total volume of 0.2 ml, containing 5 mM-ATP, 5 mM-MgCl<sub>2</sub>, 2 mM-CoA, 1 mM-dithiothreitol, 0.1 M-Tris/HCl buffer, pH 8.0, 0.15% Triton X-100, 3 mM-ciprofibrate, -nafenopin or clofibric acid, and 250–300  $\mu$ g of microsomal protein. The drugs were prepared as 10 mM solutions in 0.1 M-Tris/HCl buffer, pH 8.0,

containing 0.5% Triton X-100. After incubation at 37 °C, the reaction was stopped by the addition of 0.8 ml of chloroform/methanol (2:1, v/v). The tube was agitated vigorously for 1 min and centrifuged. The upper water/methanolic phase was used directly for the analysis of CoA thioesters by h.p.l.c. In previous experiments, it was determined that more than 97% of lauroyl-CoA was present in the upper phase, under these conditions. Similar values were obtained afterwards with the putative acyl-CoA thioesters of the drugs.

For preparative purposes, incubations were performed for 90 min at 37 °C, with 4 ml total volume of reaction mixture, under the same conditions described above. The reaction was stopped with 16 ml of chloroform/methanol (2:1, v/v). The upper phase was dried under a nitrogen stream, dissolved in 0.4–0.5 ml of water, and injected into the h.p.l.c. system in 100  $\mu$ l portions. The peak corresponding to the retention time of the derivative was collected, dried under a nitrogen stream and rechromatographed several times in the same system. The products from the three drugs were 90–95% pure, based on the h.p.l.c. peak areas. For the ciprofibrate derivative, enough material was available to perform t.l.c. A single spot was detected with iodine vapour, by using two different solvents.

Thiol groups were determined by the 5,5'-dithiobis-(2-nitrobenzoic acid) method (Ellman, 1959). CoA was determined enzymically as described by Allred & Guy (1969). U.v. spectra were obtained with a Shimadzu u.v. spectrophotometer (Shimadzu Seisakusho, Kyoto, Japan).

Ciprofibrate was kindly provided by Sterling–Winthrop Research Institute, Rensselaer, NY, U.S.A. Nafenopin (Su-13437) was a gift from Dr. F. Waetcher, Ciba–Geigy, Basel, Switzerland. Clofibric acid, co-enzymes, substrates and chemicals were from Sigma Chemical Co., St. Louis, MO, U.S.A. H.p.l.c.-grade methanol and silica gel-60 t.l.c. plates were purchased from Merck (Darmstadt, Germany).

## RESULTS

### H.p.l.c. analysis of incubation products

H.p.l.c. analysis of the reaction products, after incubation of the drugs with microsomal fractions, was first performed under conditions where the separation method resolved between ATP, CoA and the hypolipidaemic drug used as the substrate. In Fig. 1(a) the results of one such experiment are shown, with ciprofibrate as the substrate. Two reaction products were detected. One (peak 3) was formed in the absence of ciprofibrate or

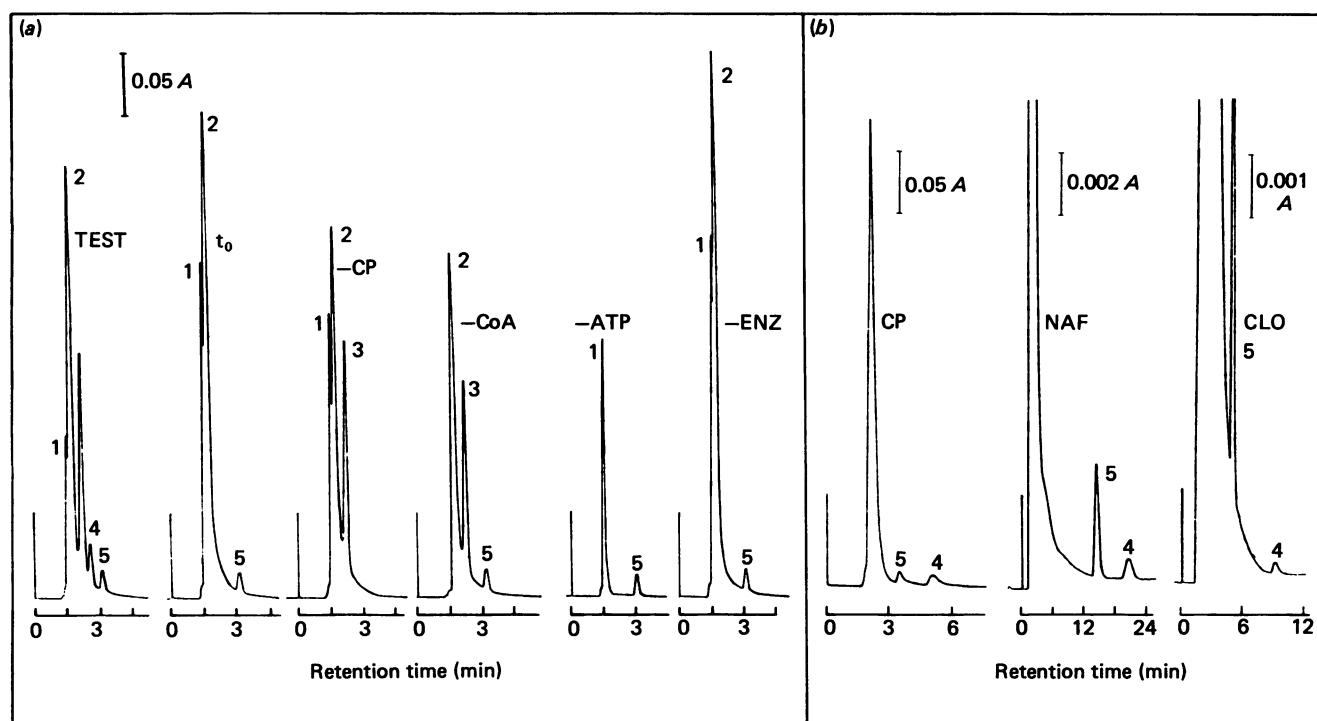


Fig. 1. H.p.l.c. chromatograms of methanol/water extracts of incubation mixtures

Drugs were incubated with microsomal fractions, CoA and ATP, and the incubation mixtures were partitioned as described in the Experimental section. (a) Chromatograms obtained with ciprofibrate as substrate. In each case, 5  $\mu$ l of the methanol/water extracts was injected. 'TEST' shows complete reaction mixture, incubated for 60 min at 37  $^{\circ}$ C;  $t_0$ , non-incubated reaction mixture; -CP, -CoA, -ATP, and -ENZ, 60 min incubation at 37  $^{\circ}$ C, respectively omitting one of the following from the reaction mixture: ciprofibrate, CoA, ATP and microsomal fraction. Peaks 1, 2 and 5 correspond to CoA, ATP and ciprofibrate respectively. The mobile phase was 55% (v/v) methanol, containing 9 mM- $\text{KH}_2\text{PO}_4$ , pH 5.5, and was pumped at a flow rate of 1.5 ml/min. Detection was at 254 nm, with 0.5 absorbance unit full scale. (b) Chromatograms obtained with ciprofibrate (CP), nafenopin (NAF) and clofibric acid (CLO) as substrates. Mobile phases used contained higher concentrations of  $\text{KH}_2\text{PO}_4$ , pH 5.5, than in (a), which causes longer retention times of drug CoA esters, and were: 55% (v/v) methanol/40 mM- $\text{KH}_2\text{PO}_4$  (ciprofibrate), 50% methanol/20 mM- $\text{KH}_2\text{PO}_4$  (nafenopin) and 40% methanol/40 mM- $\text{KH}_2\text{PO}_4$  (clofibric acid). Flow rates were 1.5 ml/min in all cases. Samples of 5, 10 and 20  $\mu$ l of the ethanol/water extracts of mixtures incubated 60 min at 37  $^{\circ}$ C were injected respectively for ciprofibrate, nafenopin and clofibric acid. Detection was at 254 nm, with 0.5, 0.02 and 0.01 absorbance unit full scale, as indicated.

CoA, and its identity was not further investigated. The other (peak 4) was tentatively identified as an acyl-CoA thioester of ciprofibrate, since its formation depended on incubation with the complete reaction mixture.

Similarly, a product formed only in the presence of CoA, ATP and microsomal fraction was detected when nafenopin or clofibric acid was used as substrate. For the three drugs it was found that increasing the phosphate concentration of the elution solvent used in the h.p.l.c. analysis resulted in a preferential retardation of the putative acyl-CoA derivative (peak 4), an observation that facilitated further purification. This effect is illustrated in Fig. 1(b). The amount of product formed (peak 4) was greater for ciprofibrate than for nafenopin or clofibric acid.

#### Alkaline-hydrolysis products of peak 4

The identity of peak 4 from the different drugs was further evaluated by studying the nature of its alkaline-hydrolysis products. Fig. 2 shows an experiment in which purified peak 4 from ciprofibrate was incubated for various times in 50 mM-KOH at 55  $^{\circ}$ C. The purified product had no detectable thiol groups, as measured by the Ellman (1959) method. Alkaline hydrolysis resulted

in a simultaneous release of thiol groups and of free CoA (Fig. 2a). It is noteworthy that the  $\mu$ mol of thiol group released, the  $\mu$ mol of free CoA liberated, and the  $\mu$ mol of the putative ciprofibroyl-CoA (measured spectrophotometrically) were coincident. Moreover, as shown in Fig. 2(b), alkaline hydrolysis resulted in a decrease of peak 4 measured by h.p.l.c., with the simultaneous appearance of two peaks; one with the same retention time as ciprofibrate (peak 5), and the other eluted in the same position as CoA (peak 1). If the amounts of these products are calculated by integrating the respective areas, and corrected by the absorption coefficients of ciprofibrate and CoA at the detection wavelength, a nearly 1:1 relation is found.

The u.v. spectra of purified peak 4 and of its hydrolysis products are shown in Fig. 3. The spectrum of peak 4 is consistent with that expected for ciprofibroyl-CoA. Furthermore, the u.v. spectrum of peak 5 is identical with that of ciprofibrate, whereas peak 1 presents a spectrum similar to, although not identical with, that of CoA. This is probably a consequence of the degradation of CoA that occurs in alkaline media (Ingebreetsen *et al.*, 1979). Similar results were obtained with purified peak 4 from clofibric acid and nafenopin.

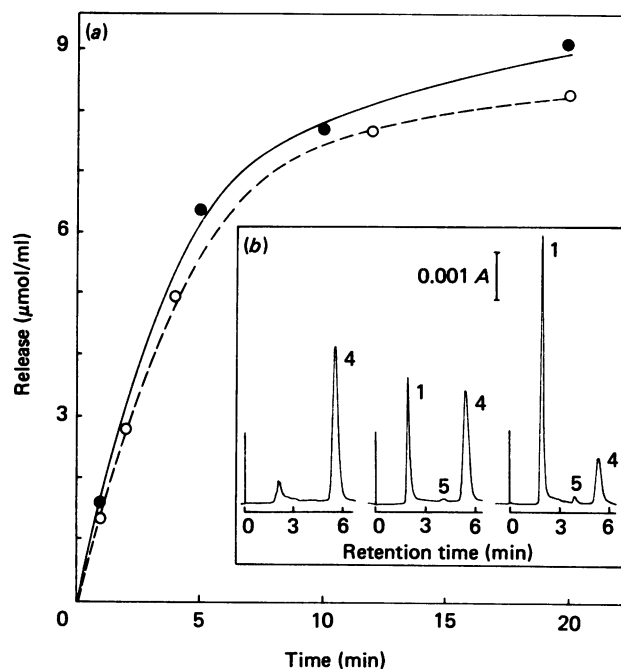


Fig. 2. Alkaline hydrolysis of the putative acyl-CoA thioester of ciprofibrate

Experiments were performed with a purified peak 4 sample, containing 9.2 μmol of the thioester/ml. This concentration was estimated spectrophotometrically at 259 nm by using an absorption coefficient of 14600 M<sup>-1</sup>·cm<sup>-1</sup>, assumed to be the same as that of CoA (Ingebretsen *et al.*, 1979). (a) Release of thiol groups (○) and CoA (●). Diluted samples of the peak-4 sample were incubated at 55 °C in 50 mM-KOH (thiol-group determination) or in 50 mM-KOH/2 mM-dithiothreitol (CoA determination). Concentrations of thiol groups [measured by the Ellman (1959) method] and of CoA (determined enzymically, and calculated by using CoA standards) are presented with corrections for initial dilutions. (b) H.p.l.c. chromatograms after alkaline hydrolysis. A 10 μM solution of the thioester containing 50 mM-KOH was incubated for 0, 3 or 7 min at 55 °C (from left to right). In each case 10 μl of the incubation mixture was injected. Mobile phase was 55% (v/v) methanol containing 50 mM-KH<sub>2</sub>PO<sub>4</sub>, pH 5.5, and was pumped at a flow rate of 1.5 ml/min. Detection was at 254 nm, with 0.01 absorbance unit full scale. Peak 5 has the same retention time as ciprofibrate, and peak 1 was eluted in the same position as CoA (solvent front).

U.v. spectra were consistent with those expected for their acyl-CoA thioesters, and alkaline hydrolysis resulted in CoA liberation, and the appearance of a product with the same retention time as the drugs (results not shown).

#### Comparative activity of microsomal acyl-CoA synthetase towards the drugs

The distribution pattern of ciprofibroyl-CoA synthetase in differential-centrifugation experiments was similar to that of palmitoyl-CoA synthetase, with more than 65% of the homogenate activity located in the microsomal fraction. As previously noted in Fig. 1(a), the activity of microsomal fractions towards ciprofibrate was higher than for nafenopin or clofibrac acid. The formation rates of CoA thioester, calculated from initial velocities from time-course curves, were 3, 0.5 and 0.04 nmol/min

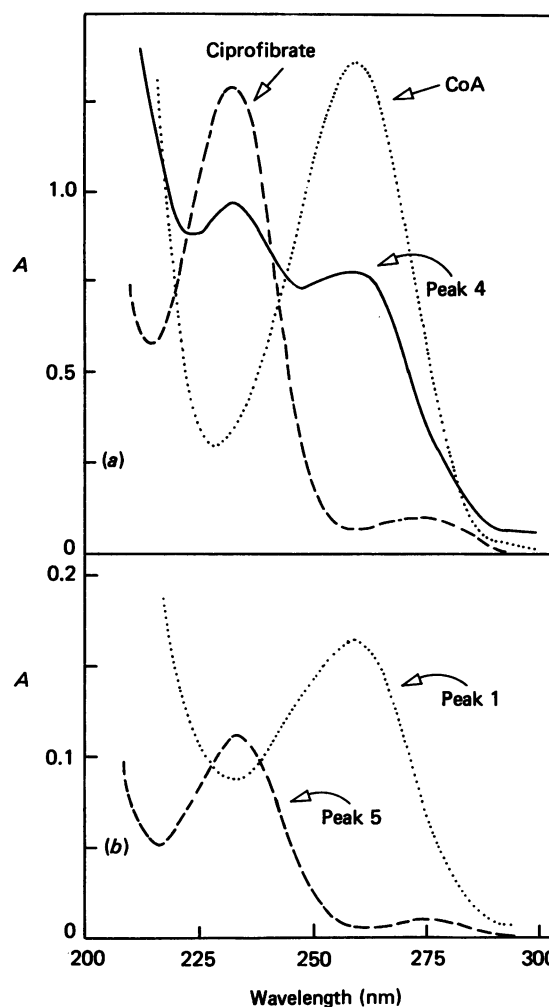


Fig. 3. U.v. spectra of the ciprofibrate derivative and of its alkaline-hydrolysis products

(a) U.v. spectra of CoA (0.1 mM), ciprofibrate (0.1 mM) and the putative acyl-CoA thioester of ciprofibrate. (b) U.v. spectra of peaks 1 and 5 isolated by h.p.l.c. Experimental conditions were similar to those reported in Fig. 2(b).

per mg of microsomal protein for ciprofibroyl-CoA, nafenopin-CoA and clofibroyl-CoA respectively (calculated on the assumption that their absorption coefficients are the same as for free CoA). Under similar assay conditions, the activity of microsomal fractions towards palmitate was 50–70 nmol/min per mg, which correlates with published data (Krisans *et al.*, 1980). Thus the order of reactivity towards the drugs is as follows: ciprofibrate > nafenopin > clofibrac acid.

In addition, ciprofibrate and nafenopin are respectively 150–200-fold and 5-fold more active as hypolipidaemic agents in the rat than is clofibrate, the ethyl ester of clofibrac acid (Best & Duncan, 1970; Arnold *et al.*, 1979). If the formation rates of the CoA esters of the three drugs are plotted against their hypolipidaemic activity relative to clofibrate, it is observed that the dosage required to produce a similar hypolipidaemic effect decreases exponentially as the capacity of microsomal fractions for activating the drugs to the corresponding acyl-CoA increases. In this comparison it is assumed that the active form of clofibrate is clofibrac acid, and that clofibrate is

easily hydrolysed to clofibric acid (see Cayen, 1983, and references cited therein).

## DISCUSSION

On the basis of the results presented here, it is proposed that the pharmacologically active species of hypolipidaemic drugs is their acyl-CoA derivative. This extrapolation to the situation *in vivo* has been confirmed by recent data showing the presence of ciprofibril-CoA in the liver of drug-treated rats (L. Amigo, M. N. Morales & M. Bronfman, unpublished work). Several considerations give additional support to this view.

First, acyl-CoA derivatives of hypolipidaemic compounds, as analogues of the natural substrates, could interfere with the various reactions in the cell that use acyl-CoA as substrates. Preliminary data from our laboratory indicate that ciprofibril-CoA is an inhibitor of acyl-CoA oxidase, the first enzyme in the peroxisomal  $\beta$ -oxidation pathway. Thus inhibition of this enzyme could be the stimulus or part of the process which leads to a compensatory reaction by the liver, namely peroxisomal proliferation. This process could be mediated by increased concentrations of natural substrates of acyl-CoA oxidase. This enzyme is induced in the liver by high-fat diets (Neat *et al.*, 1981), suggesting a substrate-induction mechanism. It is also noteworthy that hypolipidaemic agents increase preferentially the activity of peroxisomal enzymes related to lipid metabolism, whereas other peroxisomal enzymes, pertaining to other metabolic pathways, do not increase or decrease (Dallner & De Pierre, 1983, and references cited therein). This preferential increase in lipid-metabolizing enzymes induced by hypolipidaemic drugs is not restricted to peroxisomes: for example, microsomal  $\omega$ -oxidation (Orton & Parker, 1982), mitochondrial  $\beta$ -oxidation, and the cytoplasmic fatty-acid-binding protein and acyl-CoA hydrolases (Dallner & De Pierre, 1983, and references cited therein).

As well as their role as substrates, acyl-CoAs also are thought to regulate key enzymes of certain metabolic pathways (citrate synthase: Hansel & Powell, 1984; glucokinase: Tippet & Neet, 1982). The presence and/or accumulation of abnormal acyl-CoA thioesters may disrupt the control of this regulatory function.

Sequestration of CoA by the drugs could also be a possible mechanism by which hypolipidaemic drugs produce some of their effects. CoA sequestration by erucate, slowly metabolized fatty acid that induces peroxisomal proliferation (Christiansen *et al.*, 1979), has been proposed as the mechanism by which this fatty acid inhibits the CoA-dependent substrate oxidation in mitochondria from rat heart and brown adipose tissue (Christopherson & Christiansen, 1975; Alexson *et al.*, 1985). Furthermore a 2-fold increase in hepatic CoA is induced in rat liver by several hypolipidaemic compounds (Halvorsen, 1983).

A third potential area in which hypolipidaemic-drug acyl-CoA thioesters may affect cellular metabolism is through the generation of new xenobiotics.

Fears *et al.* (1978) have shown that ethyl 4-benzoyloxybenzoate (BRL 10894), a drug possessing hypolipidaemic activity, and other aryl-substituted acids

with pharmacological activity, become incorporated into triacylglycerols after incubation with liver slices. Under similar conditions no incorporation of clofibric acid was found; however, the relatively low concentration of the drug used and the short incubation periods may be important factors influencing the results. Similarly, a drug structurally related to BRL 10894 was found to form a xenobiotic cholesteryl ester (Fears *et al.*, 1982). The possible implications of these xenobiotic complex lipids are unknown, but they might have a role to play in the carcinogenic properties of hypolipidaemic drugs.

The generalization of our hypothesis to other hypolipidaemic compounds not structurally related to clofibric acid remains to be established. However, as noted above, most of them are carboxylic acids or contain groups that could be transformed to carboxylic acids. As a working hypothesis, our proposal has the main advantage that it could explain the great variety of effects induced by hypolipidaemic compounds, by one common mechanism, i.e. their activation to acyl-CoA thioesters.

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## REFERENCES

- Alexson, S. E. H., Nedergaard, J. & Cannon, B. (1985) *Biochim. Biophys. Acta* **834**, 149–158
- Allred, J. B. & Guy, G. D. (1969) *Anal. Biochem.* **29**, 293–299
- Arnold, A., McAuliff, L. G., Powers, D. K., Phillips, D. K. & Beyler, A. L. (1979) *Atherosclerosis* **32**, 155–163
- Best, M. M. & Duncan, C. H. (1970) *Atherosclerosis* **12**, 185–192
- Bronfman, M., Inestrosa, N. C., Nervi, F. O. & Leighton, F. (1984) *Biochem. J.* **224**, 709–720
- Cayen, M. N. (1983) *Prog. Drug Metab.* **7**, 173–227
- Christiansen, R. Z., Christiansen, E. N. & Bremer, J. (1979) *Biochim. Biophys. Acta* **573**, 417–429
- Christophersen, B. O. & Christiansen, R. Z. (1975) *Biochim. Biophys. Acta* **388**, 402–412
- Dallner, G. & De Pierre, J. W. (1983) *Methods Enzymol.* **96**, 542–557
- de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955) *Biochem. J.* **60**, 604–617
- Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77
- Fears, R., Baggaley, K. H., Alexander, R., Morgan, B. & Hindley, R. M. (1978) *J. Lipid Res.* **19**, 3–11
- Fears, R., Baggaley, K. H., Walker, P. & Hindley, R. M. (1982) *Xenobiotica* **12**, 427–433
- Halvorsen, O. (1983) *Biochem. Pharmacol.* **32**, 1126–1128
- Hansel, B. C. & Powell, G. L. (1984) *J. Biol. Chem.* **259**, 1423–1430
- Ingebretsen, O. C., Normann, P. T. & Flatmark, T. (1979) *Anal. Biochem.* **96**, 181–188
- Krisans, S. K., Mortensen, R. M. & Lazarow, P. B. (1980) *J. Biol. Chem.* **255**, 9599–9607
- Neat, C. E., Thomassen, M. S. & Osmundsen, H. (1981) *Biochem. J.* **196**, 149–159
- Orton, T. C. & Parker, L. G. (1982) *Drug Metab. Dispos.* **10**, 110–115
- Reddy, J. K., Azarnoff, D. L. & Hignite, C. E. (1980) *Nature (London)* **283**, 397–398
- Tippet, P. S. & Neet, X. E. (1982) *J. Biol. Chem.* **257**, 12839–12845